Biotechnology of Lactic Acid Bacteria

Novel Applications,

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Chapter 1

Updates in the Metabolism of Lactic Acid Bacteria in the Light of the "Omic" Technologies

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Lactic acid bacteria (LAB) comprise highly diverse bacterial genus and species characterized with a common feature, the ability to produce lactic acid from the fermentation of carbohydrates. Sugar metabolism of LAB together with other metabolic capacities such as proteolytic activity, production of aroma compounds and bioactive peptides, have been exploited throughout the world in the processing by fermentation of various food types, including milk, meat and plants. These bacteria are essential for the preservation of food and for maintaining and/or enhancing its organoleptic and nutritional quality.

Studies on the wide metabolic capacity of LAB considering the use of different carbon and nitrogen sources and exploring their complex regulatory circuits are the main areas of current research on these bacteria. Moreover, high-throughput technologies and the exponentially growing data bases of complete genome sequences of different LAB species, as well as all other large-scale omic-techniques combined with systems biology, bring about a constant need for updating current knowledge in LAB biology. Furthermore, it addresses strategies to exploit such knowledge into the rational design and use of LAB strains with increased industrial and consumer functionality.

1.1. Sugar Metabolism

Sugars are primary carbon and energy sources for LAB grown on substrates used for fermented food and feed production as well as in laboratory media. Many different transport systems involved in the uptake of carbohydrates, including PhosphoTransferase Systems (PTS), ATP - binding cassette (ABC), and Glycoside-Pentoside-Hexuronide transporters, exist in LAB. All LAB have the ability to utilize common hexoses but glucose is often the favored substrate. *Lactococcus lactis* imports glucose via either the mannose or cellobiose PTS and one or more

previously unknown non-PTS permease(s). GlcU were disclosed for the first time and identified as the sole non-PTS permease involved in the transport of glucose in *L. lactis* MG1363 (Castro *et al.*, 2009). The ability to ferment other sugars is strain-dependent. LAB of the milk origin can use its most abundant sugar, lactose, as a carbon source, whereas plant-associated bacteria utilize a large variety of other carbohydrates, including β-glucosides (for a review see, Aleksandrzak-Piekarczyk, 2013). Monosaccharides entering the cell or liberated in the cytoplasm by hydrolysis of disaccharides enter glycolysis at the level of glucose-6P (G6P) or are processed by the Leloir pathway (Figure 1). In *L. lactis* lactose transported by the PTS system is hydrolyzed and the galactose-6P moiety is transformed by the tagatose pathway (Tag6P), entering glycolysis at the level of triose phosphate. During the metabolism of lactose by some LAB, only the glucose moiety of lactose is fermented, while the galactose moiety is excreted into the medium, which leads to the accumulation of galactose and yealds poor-quality dairy products (Neves *et al.*, 2010; Aleksandrzak-Piekarczyk, 2013). Galactose can be imported by the non-PTS permease GalP and metabolized via the Leloir pathway (*galMKTE*). Alternatively, galactose can be imported by PTS^{Lac} (*lacFE*) and further metabolized to triose phosphates by the Tag6P pathway (*lacABCD*). A new alternative uptake route was lately discovered, consisting of galactose translocation via the galactose PTS, followed by dephosphorylation of the internalized Gal6P to galactose, which is further metabolized via the Leloir pathway (Neves *et al.*, 2010). This knowledge and the use of genetically engineered strains let for the enhancement of galactose consumption rates up to 50% in relation to that of the wild type *L. lactis* NZ9000 (Neves *et al.*, 2010).

Recently, carbohydrate fermentation profiles of twenty strains of phenotypic *L. lactis* subsp. *lactis* belonging to the *lactis* and *cremoris* genotypes were analyzed by the fenotypic and genotypic methods showing the strain-to-strain variations (Fernandez *et al.*, 2011). These two

groups of strains showed distinctive carbohydrate fermentation and enzyme activity profiles, with the strains in the *cremoris* group showing broader profiles.

Fermentation of sugars leads to the formation of lactic acid as major end product or in combination with other organic acids and ethanol. Three types of carbohydrate metabolism can be distinguished in LAB based on the characteristics of the metabolic products: homofermentation, mixed-acid metabolism and heterofermentation (Figure 1). General characteristic of these fermentation types and their regulation modes have been comprehensivly described in the first edition of this book (Mayo *et al.*, 2010) and the references within. The control and regulation of the glycolytic flux in LAB is still not fully understood (Martinussen *et al.*, 2013). It has been demonstrated that the control of the glycolytic flux in *L. lactis* cannot be explained neither by the single enzyme nor single sugar transport bottleneck. The control was not in the ATP demand either, however it cannot be rule out that regulation of glycolytic flux is the combination of these mechanisms (Martinussen *et al.*, 2013).

The metabolism of mono- and disaccharides is well understood; however, few data are available on the metabolism of higher oligosaccharides which are abundant in many habitats such as cereals, milk, fruits, and the upper intestine of animals. Moreover, the research interest has shifted from (digestible) disaccharides to (indigestible) higher oligosaccharides with the development of intestinal microbial ecology as well as the increase in commercial application of prebiotic oligosaccharides. Oligosaccharide metabolism has been discussed in detail for four major groups of compounds (i) starch, maltodextrins, and isomaltooligosaccharides (IMO); (ii) fructo-oligosaccharides (FOS); (iii) β-galacto-oligosaccharides (βGOS); (iv) raffinose-family oligosaccharides (RFO) as well as α-galacto-oligosaccharides (RFO and αGOS, respectively) (Gänzle & Follador, 2012).

LAB are able to direct some part of the sugar pool toward biosynthesis of EPS. These long - chain saccharides can be loosely attached to the cell surface forming some kind of capsules or are secreted to the environment. EPS represent molecules with different structures, sizes, and sugar composition. They are classified into two groups: homopolysaccharides (HoPS) consisting of one type of monosacharide (α-D-glucans, β-D-glucans, fructans, and others represented by polygalactan) and heteropolysaccharides (HePS) composed of different types of monosaccharides, mainly D-glucose, D-galactose, L-rhamnose, and their derivatives. There has been an increasing interest in the EPSs produced by LAB during the last decade. The EPS production by Lactobacilli has been discussed in the comprehensive review (Badel, Bernardi & Michaud, 2011). Since the first eddition of this book several novel works concerning a diversity of biopolymers produced by cereal associated (Bounaix *et al.*, 2009, 2010; Palomba *et al.*, 2012) and intestinal LAB (Salazar *et al.*, 2009; Górska-Frączek *et al.*, 2011, 2013; Sims *et al.*, 2011) have been presented.

HoPS are synthesized extracellulary by highly specific glycosyltransferase enzymes, glucan or fructan sucrases. This process requires sucrose as a specific substrate and the energy that comes from its hydrolysis. Glucansucrase enzymes, their recently elucidated crystal structures, their reaction and product specificity, and the structural analysis of α-glucan polymers have been described in the recent review (Leemhuis *et al.*, 2013). EPS belonging to HePS are synthesized from glucose, galactose, or other monosaccharides by a combined action of different types of glycosyltransferase enzymes. Four major consecutive steps of EPS biosynthesis in LAB involve sugar transport into the cytoplasm, synthesis of sugar-1P, polymerization of repeating unit precursors, and finally EPS export outside the cell. It is postulated that regulation of EPS production can be possible at each of the different steps during biosynthesis. Synthesis of two EPSs in *Lactobacillus johnsonii* FI9785 is dependent on the identified 14-kb *eps* gene cluster;

however, the precise regulation of the biosynthesis of individual EPS has yet to be identified (Dertli *et al.*, 2013). Genome sequencing yields the extensive information of EPS-related genes and their organization (Koryszewska-Baginska, Bardowski & Aleksandrzak-Piekarczyk, 2014) which provides a basis for future experiments and disclose putative applications of LAB strains.

1.1.1. Practical Aspects of Sugar Catabolism

The ability of LAB to ferment sugars has been widely utilized in various food production processes. Apart from the role in food industry they have a potential to be used for production of value added products such as biofuels and biochemicals because of their robustness and tolerance to ethanol, low pH and high temperature (Martinussen *et al.*, 2013). Nowadays, great attention has been directed to optimization of lactate production from natural substrates like so-called starchy or lignocellulosic materials from agricultural, agro-industrial, and forestry sources due to their abundance, low price, high polysaccharide content, and renewability; for a review, (Okano *et al.*, 2009; Abdel-Rahman, Tashiro & Sonomoto, 2011; Castillo Martinez *et al.*, 2013). As already mentioned, LAB can direct part of the sugar pool toward biosynthesis of EPS. These microbial polysaccharides have been used in the food industry as emulsifiers, thickeners, viscosifiers, and stabilizers. EPS from LAB play an important role in the reology and texture of fermented milks (Ramchandran & Shah, 2009) and other fermented products, such as sourdough used for baking purposes (Katina *et al.*, 2009; Galle *et al.*, 2010) or even cereal-based beverages (Zannini *et al.*, 2013). Apart from the studies on the wheat and rye dough and bread there has been effords toward the improvement of the quality, safety and acceptability of gluten-free bread (Moroni, Dal Bello & Arendt, 2009) and the replacement of hydrocolloids by the EPS produced by LAB in sorghum sourdough (Galle *et al.*, 2011). EPS from LAB are of great interest for Agro-Food industries since they have a vast structural diversity, which opens the way to innovations.

However, because of the low production of polysaccharides by the majority of LAB species the optimized methodologies of EPS production and recovery are still required to facilitate their potential applications (Notararigo *et al.*, 2013). EPS derived from LAB play also a crucial role in conferring beneficial physiological effects on human health, such as antitumour activity, immunomodulating bioactivity and anticarcinogenecity (Patel, Majumder & Goyal, 2011). Recently, the antitumour effects have been presented for cell-bound EPS isolated from *Lactobacillus acidophilus* 606 (Kim *et al.*, 2010) and *Lactobacillus plantarum* 70810 (Wang *et al.*, 2014). EPSs have been shown to exhibit immunomodulatory activity with professional immune cells, such as macrophages (Liu *et al.*, 2011; Ciszek-Lenda *et al.*, 2011) but also with intestinal epithelial cells (Patten *et al.*, 2014). EPSs produced by strains of *Lactobacillus reuteri* inhibited enteroxigenic *Escherichia coli*-induced hemagglutination of porcine erythrocytes thus confirming their therapeutic potential (Wang, Ganzle & Schwab, 2010). The EPS isolated from *Lb. plantarum* 70810 was shown to have a metal binding capacity and may be used as a potential biosorbent for the removal of lead from environment (Feng *et al.*, 2012). LAB can also produce a variety of functional oligosaccharides which have huge industrial applications as prebiotics (Pepe *et al.*, 2013), nutraceuticals, sweetners, humectans, drug against colon cancer, immune stimulators (Patel, Majumder & Goyal, 2011). Furthermore, some probiotic LAB can utilize prebiotic compounds such as non-digestible fructooligosaccharides (FOS), inulin-type fructans or recently studied β-glucans (Russo *et al.*, 2012) which stimulate the growth of beneficial commensals in the gastrointestinal tract.

1.2. Citrate Metabolism and Formation of Aroma Compounds

In addition to sugars, several LAB species have the capability to metabolise citrate. Citrate fermentation by LAB leads to the production of volatile compounds such as diacetyl, acetoin and

butanediol (C_4 compounds) that are responsible for the typical aroma of many fermented dairy products. Therefore, selected metabolising citrate LAB strains, such as *L. lactis* subsp. *lactis* biovar. diacetylactis (*L. diacetylactis*) and some species of the genera *Leuconostoc* and *Weissella* are currently in use as starter and adjunct cultures to lead production of C_4 aroma compounds. However, in some other fermented products, such as wine, beer and sausages, these volatile compounds are considered off-flavours and their presence should be avoided. The knowledge of citrate utilization by LAB have been described in details by Quintans et al. and summarized in the first edition of this book (Quintans *et al.*, 2008; Mayo *et al.*, 2010). Therefore, in this chapter we present citrate metabolism from the perspective of current achievements.

1.2.1. Citrate Transport

Transport of citrate, which constitutes a limiting step for citrate utilization, is performed by a variety of membrane-associated permeases. In contrast, volatile compounds formed in the cytoplasm are secreted without requiring specific transporters. Most LAB species internalise citrate by a 2-hydroxycarboxylate type of transporters (2-HCT transporter family), which transports dicarboxylic and tricarboxylic acids. The family includes CitP from *Lactococcus*, *Leuconostoc* and *Weissella* species (Pudlik & Lolkema, 2010), an antiport transporter exchanging H-citrate^{2-} and lactate^{1-}, generating a membrane potential (Figure 2). In *L. diacetylactis* CitP is encoded within the *citQRP* operon located on so-called citrate plasmid (Drider, Bekal & Prévost, 2004; Kelly, Ward & Leahy, 2010a). Plasmid genes are complemented with chromosomally-encoded citrate catabolism *citM-citCDEFXG* cluster (Quintans *et al.*, 2008). In *L. diacetylactis*, specific transcriptional activation of the promoters controlling *cit* operons takes place at low pH and provides an adaptive response to acidic stress as it has been confirmed by transcriptomics analysis of *L. diacetylactis* growing in milk (Raynaud *et al.*, 2005) and under cheese conditions

(Cretenet *et al.*, 2010). In *Weissella paramesenteroides* and *Leuconostoc mesenteroides* subsp. *cremoris*, the *citP* genes (*citMCDEFGRP*) are included in respectively plasmidic or chromosomal *citI* cluster (Martín *et al.*, 1999; Martín *et al.*, 2000; Bekal *et al.*, 1998). Enlarged citrate plasmids (15 to 23 kbp) have recently been observed in wild (non-starter) strains of *L. diacetylactis* (Drici *et al.*, 2010; Kelly, Ward & Leahy, 2010a).

In contrast, in *Enterococcus faecalis* and *Lactobacillus casei* citrate is transported by CitM and CitH transporters, respectively, belonging to the family of citrate-metal symporters (CitMHS) (for a review see, Lensbouer & Doyle, 2010). Citrate by these types of transporters is transported in complexes with cations (either Ca^{2+}, Mn^{2+} or Fe^{3+}). Recent characterization of the citrate transport in *Lb. casei* has revealed that CitH is a proton symporter of citrate in complex with Ca^{2+}, which is also the actual metabolic substrate (Mortera *et al.*, 2013).

Other type of citrate transporters may be found in *Lb. plantarum, O. oeni* (reviwed by Mayo *et al.*, 2010) and atypical citrate-fermenting wild strain of *L. diacetylactis* (Passerini *et al.*, 2013b).

1.2.2. Conversion of Citrate into Pyruvate and Production of Aroma Compounds

Once inside, citrate is converted into acetate and oxaloacetate in a reaction catalysed by the citrate lyase (CL) enzyme complex (Figure 2).

The second step of citrate metabolism is the decarboxylation of oxaloacetate, which is catalysed by oxaloacetate decarboxylase (OAD) generating pyruvate and CO_2 (Figure 2). Analysis of the genomes of various LAB species has shown genes encoding the α-, β- and δ-subunits of the OAD (Makarova *et al.*, 2006). However, the physiological role of the OAD complex remains poorly studied in this bacterial group. Only the enzymes from *E. faecalis* (Repizo *et al.*, 2013) and *Lb. casei* (Mortera *et al.*, 2013) have been subjected to recent investigation. In other LAB species, such as *L. diacetylactis, W. paramesenteroides*, *Leuc. mesenteroides*, *Lb. plantarum* and

O. oeni, the decarboxylation of oxaloacetate is catalyzed by a soluble cytoplasmic, single-subunit OAD enzyme which is a malic enzyme (ME) (Sender *et al.*, 2004). Surprisingly, the *cit* locus of *E. faecalis* has been recently shown to contain genes coding for both OAD and ME types of oxaloacetate decarboxylases (Espariz *et al.*, 2011).

Metabolism of pyruvate can yield in LAB different end-products such as lactate, formate, acetate, and ethanol, but also some important aroma compounds such as diacetyl, acetoin, and butanediol (Neves *et al.*, 2005).

1.2.3. Conversion of Citrate into Succinate

Some LAB species cannot truly convert citrate into pyruvate. The route to succinate via malate and fumarate is characterized by the CitT transporter that takes up citrate in exchange for the end product succinate. Furthermore, complete TAC pathway has recently been identified in *Lb. casei* by *in silico* analysis of its genome (Díaz-Muñiz *et al.*, 2006). The dominant end products of citrate metabolism in this bacterium are acetic acid and L-lactic acid, both under excess and limiting amounts of carbohydrates. Trace amounts of D-lactic acid, acetoin, formic acid, ethanol, and diacetyl, thus confirming the activity of both OAD and complex enzymes. However, production of succinic acid, malic acid, and butanendiol was not observed (Díaz-Muñiz *et al.*, 2006; Mortera *et al.*, 2013).

1.2.4. Bioenergetics of citrate metabolism

The co-metabolism of glucose and citrate produces different physiological effects in homofermentative and heterofermentative LAB. In homofermentative LAB citrate utilization has a protective effect against acid stress, whereas in heterofermentative LAB “citrolactic” fermentation generates one extra mol of ATP per mol of citrate. During growth in milk, *L. diacetylactis* metabolises lactose producing lactic acid, which is exchanged for citrate during

excretion by the antiporter CitP. As a homofermentative organism *L. diacetylactis* converts glucose into lactate producing 2 moles of ATP per mol of glucose metabolised. The NAD^+ consumed in the first steps of the pathway is regenerated during the transformation of pyruvate into lactate; thus maintaining the redox potential. In the presence of glucose and citrate, each mol of citrate produces one mol of pyruvate without generating NADH. This excess of pyruvate is diverted to the synthesis of α-acetolactate and, subsequently, to the production of aroma compounds. As in *L. diacetylactis*, the higher biomass attained by *Lb. casei* during co-metabolism of Ca^{2+}-citrate and carbohydrates has been considered to be due to a counteraction of growth inhibition by acidification at the final stages of carbohydrate metabolism (Mortera *et al.*, 2013).

1.3. Proteolytic System of Lactic Acid Bacteria

The *L. lactis* strains isolated from dairy environments are characterized by a high number of amino acid auxotrophies and the presence of properties that allow the utilization of milk proteins as a source of amino acids. These properties are common to dairy strains originating from distant geographic locations in Asia, Europe, North America, and New Zealand (Rademaker *et al.*, 2007; Kelly, Ward & Leahy, 2010b).

LAB use the proteins of the media as source of amino acids, and this explains the importance of their proteolytic system since LAB are dependent on it to obtain the essential amino acids. Beside of this, amino acids are not only the building blocks for proteins and peptides, they also serve as precursors for many other biomolecules. Being precursors of aromatic compounds, amino acids are important for the final flavor of food products. Other molecules with different biological activities related to the probiotic properties of LAB (antihypertensive peptides), are also consequence of the proteolytic LAB activity.

Proteolytic system can be divided into several steps: protein degradation, peptides transport, peptide degradation and the last step amino acid catabolism (Figure 3).

1.3.1. Protein Degradation.

The study of the protein breakdown by LAB was initially focused on the ability to degrade casein and *L. lactis* was the first organism studied as a model. The hydrolysis of caseins by LAB is initiated by a cell-envelope proteinase (CEP), which degrades proteins into oligopeptides. Moreover, gene deletion studies have shown that LAB are not able to grow in milk in the absence of a functional CEP. However, since the protease is located extracellularly, peptides produced by it will also be consumed by protease-negative cheater variants, allowing these to invade the culture (Bachmann *et al.*, 2012).

Lactocepins comprise a broad group of CEP which belongs to the family of subtilisin and are serine proteases. CEPs are anchored to the cell wall via a mechanism involving the typical sortase A (SrtA) (Dandoy *et al.*, 2011). Depending on the respective species or even strain, LAB lactocepins are encoded by *prtP*, *prtB*, *prtS* and/or *prtH*, which differ from each other in the number of functional domains that exhibit.

Most of the LAB possesses only one CEP but there are exceptions. In *Lactobacillus helveticus* CNRZ32 four CEP genes (*prtH*, *prtH2*, *prtH3*, and *prtH4*) have been found (Jensen, Ardö & Vogensen, 2009). In contrast, to the highly conserved peptidase sequence, the CEP distribution differs widely among strains. The most abundant paralog is *prtH*3, which is carried by over 80% of the strains tested followed by *prtH* and *prtH4* paralogs (Broadbent *et al.*, 2011). The presence of several genes encoding the protease of *Lb. helveticus* could explain the high efficiency of its proteolytic system. From the identified genes, only *prtH2* is common to all the characterized strains of *Lb. helveticus* (Genay *et al.*, 2009). Analysis of the proteinase from BGRA43 showed

that the only active gene was *prtH*. In *Lb. helveticus* as in other LAB species, CEP activation requires a maturation proteinase termed PrtM. In *Lb. helveticus* CNRZ32 two PrtMs (PrtM1 and PrtM2) have been identified (Savijoki, Ingmer & Varmanen, 2006). Further studies (Genay *et al.*, 2009; Broadbent *et al.*, 2011) have reported that PrtM1 is required for activation of PrtH and PrtM2 plays a role in activation of other CEP paralogs in *Lb. helveticus*.

As it has been previously indicated, the CEP activity was initially evaluated against casein as substrate. However, during the last years LAB strains have been isolated from environments different from milk or fermented products and also exhibited casein hydrolytic activity. *Lb. helveticus* BGRA43 isolated from human feces has also a strong proteolytic activity and is able to completely hydrolyze α_{s1}-, β-, and κ-caseins (Strahinic *et al.*, 2013). Von Schillde *et al*. demonstrated that the lactocepin secreted by *Lactobacillus paracasei* VSL#3, selectively degrades cell-associated, and tissue distributed IP-10 and other proinflammatory chemokines in vitro (von Schillde *et al.*, 2012). These findings confirm that this *prtP* encoded lactocepin is highly selective protease although it targets a broad spectrum of cleavage sites, indicating that additional protein characteristics like surface charge and/or the three dimensional structure determine whether a protein is cleaved or not. The selective degradation of proinflammatory chemokines could be also related to differences in the regulation of *prtP* expression.

In *Streptococcus thermophilus*, the cell wall-associated proteinase PrtS, is highly conserved (95% identity) with the PrtS of *Streptococcus suis*. Although recent work suggested that PrtS contributes to the virulence of *S. suis* (Bonifait *et al.*, 2010), it is well established that in *S. thermophilus* strains, the primary role of PrtS involves the cleavage of casein to oligopeptides, a clear function related to its dairy adaptation. The analysis of the distribution of the *prtS* gene in *S. thermophilus* showed that the gene is infrequent in historical collections but frequent in recent industrial ones. Moreover, this "ecological" island conferring an important metabolic trait for

milk adaptation appears to be disseminated by lateral transfer in the *S. thermophilus* population. Taken together, these data support an evolutionary scheme of *S. thermophilus* where gene acquisition and selection by food producers are determining factors (Delorme *et al.*, 2010).

The second stage of protein degradation is the transport of di-, tri-, and oligo-peptides into the cell by different peptide transport systems. Three oligo-, di- and tri-peptide transport systems, (Opp, Dpp and DtpT, respectively) has been described.

Lb. acidophilus, *Lactobacillus brevis*, *Lb. casei* and *Lactobacillus rhamnosus*, like *L. lactis*, possess all these three LAB peptide transport systems. In *Lb. helveticus*, genome analysis revealed that some strains such as DPC4571 have three peptide transport systems and others like H10 has two, Opp and DtpT systems. These results indicate that the proteolytic system may differ even between different strains from the same species. In contrast, *Lb. reuteri* has only one functional peptide transport system, the DtpT (Liu *et al.*, 2010).

1.3.2. Peptidases

After the casein-derived peptides are taken up by the LAB cells, they are degraded by a concerted action of peptidases with differing and partly overlapping specificities. Peptidases are a very important part of the proteolytic system in LAB since they are involved in the hydrolysis of peptides and the release of essential amino acids. Peptidases can be divided initially in two groups, endopeptidases, which hydrolyze internal peptides bonds from the oligopeptides generating peptides which can be substrate of the exopeptidases and exopeptidases which act on the terminal ends of the oligopeptides, generating smaller peptides or amino acids.

The main endopeptidases characterized in LAB are PepO, PepF, PepG and PepE. All of them act on NH_2-X_n↓X_n-COOH substrates.

PepO is encoded by three paralogous genes (*pepO, pepO2* and *pepO3*). The PepO gene content of *Lb. helveticus* strains is identical, however strain heterogeneity may be observed due to the loss of gene function or sequence polymorphisms that affect the specificity or relative activity of individual peptidase enzymes (Broadbent *et al.*, 2011).

Three paralogous genes have been described to encode PepF: *pepF*, *pepF1* and *pepF2*. In *L. lactis*, *pepF1* is located on the chromosome, while *pepF2* is on a plasmid. This location explains the observed variability of absence or presence of this gene in different *Lactococcus* strains (Liu *et al.*, 2010).

PepE and PepG proteins are absent in lactococi and streptococci. In *Lactobacillus*, two paralogous genes have been described for PepE (*pepE* and *pepE2*). In *Lb. helveticus*, these genes were almost universally conserved among strains from different origin including cheese, whey, whiskey malt and commercial cultures strains. The encoded enzyme plays also an important role on the debitterness *Lb. helveticus* function (Broadbent *et al.*, 2011).

Exopeptidases are traditionally classified according to their specificity. In LAB, four groups have been established: aminopepdidases, dipeptidases, tripeptidases and proline specific peptidases. Aminopeptidases hydrolyze one amino acid from the N-terminal oligopeptide NH_2–X↓Xn–COOH. It can be divided in two groups, general aminopeptidases and specific aminopeptidases. General aminopeptidases (PepN and PepC), are present in all genomes, regardless of origin strain, usually with one gene per genome. Some LAB genomes have two peptidase homologs, possibly with the same function, e.g. two PepC homologs (GI codes: 42518641 and 42518638) in *Lb. johnsonii* (Liu *et al.*, 2010).

Specific aminopeptidases are divided attending to the type of the residue hydrolyzed. PepS, has been only described in *S. thermophilus* strains and is specific for aromatic residues. PepA specific for Glu and Asp residues is present in streptococci and in some *Lactobacillus* and *Lactococcus*

strains and is absent in *Pediococcus* and *Oenococcus*. PepM is specific for methionone residues and is present in *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Oenococcus* strains (Liu *et al.*, 2010).

Dipeptidases are exopeptidases specific for dipeptides NH2–X↓X–COOH. The PepD dipeptidase family has a broad specificity towards various dipeptides. The *pepD* genes are distributed heterogeneously in LAB genomes, varying from 0 to 6 paralogs.

PepV is also encoded by multiple paralogous genes and is present in all the LAB analyzed genomes, has also a broad specificity.

Tripeptidases, are enzymes that release an amino acid from the N-terminal position of tripeptides NH2–X↓X–X–COOH. They have a broad specificity, although have a preference for hydrophobic peptides and do not hydrolyze proline residues. Only one enzyme has been characterized in this group, PepT. The *pepT* gene is present in all the sequenced LAB genomes and in some of them with two paralogous genes (*Lb. acidophilus*, *Lb. jhonsonii* and *Lactobacillus gasseri*).

Proline specific peptidases can hydrolyze proline residues from N-terminal position like PepI or proline iminopeptidase which possesses aminopeptidase activity toward N-terminal proline peptides, preferably tri-peptides (NH2–Pro↓Xn–COOH), while prolinase PepR has a broad specificity for dipeptides (NH2–Pro↓X–COOH). Genome sequence analysis revealed that these proteins are absent in all *L. lactis* strains. However, the activity of cell extract of *Lb. helveticus* and *Lb. rhamnosus* toward several proline dipeptides was significantly reduced in a PepR-deletion mutant. Those observations suggest that PepI and PepR may contribute specifically to the proteolytic capacity on proline-containing peptides of *Lactobacillus* strains (Liu *et al.*, 2010).

PepP is a member of the proline peptidases, which cleave off any N-terminal amino acid linked to proline in an oligopeptide (NH2–X↓Pro–Xn–COOH). PepQ is also a proline peptidase, however

specific for Pro dipeptides (NH2–X↓Pro–COOH). In the PepP subgroup, one gene is found in each LAB genome except in *Lactobacillus sakei* and *Pediococcus pentosaceus*. The absence of the *pepP* genes in both genomes is very likely due to a gene loss event. Genes from the PepQ cluster are distributed equally in all LAB genomes, generally as one copy per genome. However, the *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) strains have two *pepQ* paralogs. One paralog is clustered with the other orthologs of LAB, whereas the second paralog is located in a separate cluster. This might be the result of an ancient duplication or horizontal gene transfer (HGT) event. PepX is an endopeptidase enzyme specific for proline residues (NH2–X–Pro↓Xn–COOH). It is present in all genomes, usually with one gene per genome. Although some LAB genomes have two peptidase homologs, mainly strains from dairy origin and possibly with the same function. PepX2 is a PepX homolog of *L. lactis* subsp. *lactis* IL1403. This putative *pepX2* gene was originally annotated as a hypothetical protein named *ymgC*. The only members of the PepX2 (YmgC) group in sequenced LAB genomes are from *L. lactis* subsp. *lactis* IL1403 and *Pediococcus* (Liu *et al.*, 2010).

1.3.3. Technological Applications of Proteolytic System

Proteolytic system provide to LAB all the amino acids required to grow. For instance, *Lb. bulgaricus* and *Lb. helveticus* have a very extensive set of proteolytic enzymes, which is consistent with previous knowledge that *Lb. bulgaricus* serves as the proteolytic organism in yoghurt rather than *S. thermophilus*. *Lb. helveticus* is a proteolytic cheese adjunct culture that has been used to degrade bitter peptides in cheese. Interestingly, *Lb. bulgaricus* encodes the Dpp system with preference for uptake of hydrophobic di/tripeptides, complementing *S. thermophiles*, which encodes the general di/tripeptide transporter DtpT in its genome, suggesting that more peptides can be utilized by both bacteria when grown together. LAB species of plant origin, such

as *Lb. plantarum*, *Oenococcus oeni*, and *Leuc. mesenteroides*, encode less proteolytic enzymes in their genomes, which agree with their ecological niche that is fiber-rich but contains less protein. Beside of this, milk proteins have been used as a raw material to obtain bioactive peptides. Various peptides with physiological functions such as immunostimulating, antimicrobial, opioid, anti-cancer, mineral binding and antihypertensive peptides have been isolated as product of the proteolytic activity of LAB (Griffiths & Tellez, 2013).

1.3.4. Amino Acid Catabolism

The catabolism of amino acids has implications with regard to the quality (formation of flavor compounds) and safety (biogenic amines synthesis) of fermented foods. Amino acid catabolism is also believed to have an important role in their ability to obtain energy in nutrient-limited conditions and in some cases has been also identified as a mechanism of pH control.

Amino acid degradation is an important key for volatile compounds synthesis and the transamination of some amino acids, branched chain methionine and aromatic aminoacids. The latter one is the main pathway for degradation leading to formation of α-keto acids, which in turn are degraded to various aroma compounds. The conversion of amino acids to keto- and hydroxyl acids is initiated by *Lactobacilli*, while *Lactococcus* strains further convert these products to carboxylic acids. This cooperation between LAB and NSLAB leads to an enhanced cheese flavor.

Leucine, valine and isoleucine catabolism can be divided into two parts. The first part constitutes the main degradation pathway, and is performed by an aminotransferase reaction, which converts the amino acid to alpha-keto acid or alpha-keto isocaproate (KICA) in the case of leucine. Then, this compound can be converted to aldehyde, alcohol or carboxylic acid following three different pathways, an alpha-keto acid decarboxylation via, an oxidative decarboxylation or an alternative dehydrogenation route resulting in α-hydroxy-isocaproate (HICA) (Smit, Smit & Engels, 2005).

Aromatic amino acids are also degradated by amino transferase enzymes. Transamination leads to indole pyruvate, phenyl pyruvate and p-hydroxy-phenyl pyruvate from Trp, Phe and Tyr, respectively. This reaction is catalyzed by the aromatic aminotransferase AraT.

Alpha-ketoacids resulting from aromatic amino acid transamination are further degraded to various compounds by enzymatic (dehydrogenation, decarboxylation, oxidative decarboxylation) or chemical reactions.

Sulphur compounds, which are produced by sulphur amino acid catabolism are potent odorants contributing to flavour in many fermented foods.

Methionine catabolism gives rise to various volatile sulfur compounds (VSCs) such as H_2S, methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) (Fernández *et al.*, 2000). Recently, the screening of LAB strains isolated from raw goats' milk cheeses for the major enzymes critical to the formation of VSCs from l-methionine showed a large biodiversity in enzyme capabilities. In this study, lactococci displayed higher activities of two enzymes (C–S lyase and aminotransferase) specific towards the sulphur-containing compounds than did *Lactobacillus* and *Leuconostoc* (Hanniffy *et al.*, 2009).

Besides flavor compounds, amino acid catabolism gives rise to biogenic amines (BA). BA are organic basic nitrogenous compounds with biological activity that are mainly formed by decarboxylation of amino acids. BA are present in a wide range of foods, including dairy products, which can accumulate at elevated concentrations. Consumption of food containing high amounts of these amines has toxicological effects. BA are mainly formed by amino acid decarboxylation. Tyrosine, histidine, lysine, ornithine and arginine are decarboxylated to produce tyramine, histamine, cadaverine, putrescine and agmatine, respectively. Agmatine could be deaminated to produce putrescine.

The decarboxylation pathways involve, the transport of the amino acid into the cell, the decarboxylation, and the way out of the cell of the BA formed. The interchange amino acid/BA is performed by a transporter protein. Tyramine, histamine and putrescine biosynthesis pathways have been characterized in LAB. The genes encoding the decarboxylation enzyme and the transporter proteins are located in most of the strains on chromosomes. Although the ability to produce BA in LAB has been proposed as a strain characteristic, the genome analysis of *E. faecalis*, *Enterococcus durans*, and *Enterococcus faecium* revealed that in the case of tyramine, this ability is species specific (Ladero *et al.*, 2012).

Tyramine biosynthesis requires the presence of tyrosine, the decarboxylating enzyme (TDC) and a transporter protein (TyrP) to interchange tyrosine/tyramine. Genes encoding these proteins form a cluster on the chromosome of the producer strain. A third open reading frame, encoding a protein homologous to tyrosyl tRNA synthase is always present in these clusters, upstream of the *tdc* gene (Linares *et al.*, 2012). Strains from *Lactobacillus* and *Enterococcus* genera are the main tyramine producers in cheese.

In the case of the synthesis of putrescine in dairy products, the agmatine pathway (AgDI) has been proposed as the main route, since ornithine decarboxylase pathway is not present in LAB from dairy origin. These bacteria make putrescine from agmatine in three steps catalyzed by agmatine deiminase (AgDI), putrescine transcarbamylase (PTC), and carbamate kinase (CK). An antiporter exchanges putrescine for agmatine. Apart from puterscine, ATP and NH_3 are also products of these reactions. This pathway has been identified in *E. faecium*, *E. faecalis*, *Lb. brevis* and *Lactobacillus curvatus*. In these two last species, tyramine and putrescine pathways are linked to the chromosome. Ladero *et al*., demonstrated the capability of some *Lactococcus* strains to produce putrescine from agmatine (Ladero *et al.*, 2012). In this species, some of the strains

carry the cluster, other have an insertion element that makes the cluster non-functional and some strains do not have the genes. These combinations suggest a putative evolution from an ancestral strain carrying the genes, which could accumulate mutations or even lose the genes if agmatine is not present in the strain environment.

The agmatine pathway is similar to the arginine deimanse pathway (ADI), which comprises three reactions catalyzed by arginine deiminase, ornithine carbamoyl-transferase and carbamate kinase. The ADI pathway is widely distributed among LAB, it has been described in strains belonging to genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus* and *Weissella*.

Histamine is the another BA very frequent and abundant in fermented products. It is produced from histidine in the reaction catalyzed by the enzyme histidine decarboxylase (HdcA). An antiporter protein which interchange histidine to histamine is also required for the histamine synthesis. The analysis of the histamine cluster revealed the presence of a gene coding for the HdcB protein, which is cotranscribed with *hdcA*, and is necessary for the HdcA maturation (Trip *et al.*, 2011). A gene enconding a protein homologous to histidyl tRNA synthetase is also located in this cluster although its position varies from one species to another (Calles-Enriquez *et al.*, 2010). *Hdc* cluster has been characterized in different species of *Lactobacillus*, *O. oeni*, *S. thermophilus*, *Tetragenococcus muriaticus* and *Tetragenococcus halophilus*. With the exception of *Lactobacillus hilgardii*, this cluster is located on the chromosome (Lucas *et al.*, 2005).

The analysis of the conditions required to BA synthesis shows that the presence of amino acid is always necessary however, other parameters such as pH, carbon source or temperature change from one BA to another (Linares *et al.*, 2012).

1.4. Metabolism of LAB in the Light of Genomics, Comparative Genomics and Meta-genomics

The use of next generation sequencing technology revolutionized the microbial science by the rapid increase in the number of genomes available within the public databases. Since the first published LAB genome (Bolotin *et al.*, 2001) nearly 100 complete genomes from typical LAB species (excluding enterococcides) have been deposited in the NCBI database and more than 400 whole genome sequencing projects with the status permanent draft or incomplete have been reported according to the GOLD database (as on 17 February 2014). Among draft genome sequences a dozen of *Lactobacillus* strains have been generated as part of the Human Microbiome Project (The Human Microbiome Jumpstart Reference Strains Consortium *et al.*, 2010). The technological development and the reduction of sequencing costs enabled the resequencing of the *L. lactis* MG1363 genome and the sequence comparison with the genome of its derivative (the *L. lactis* NZ9000 containing "NICE" system for nisin-controled protein overexpression) (Linares, Kok & Poolman, 2010). This study revealed six differences, which were not due to the errors in the published sequence. Two specific mutations were localized in a region of the *ptcC* promoter with the key role in the regulation of cellobiose and glucose uptake. The comparative analysis, based on six *Lactobacillus* and several other genomes of LAB identified nine niche-specific genes (O'Sullivan *et al.*, 2009). Comparative analysis of 20 complete *Lactobacillus* genomes showed that their pan genome consists of approximately 14,000 genes and indicated the presence of a core genome of 383 orthologous genes (Kant *et al.*, 2011). The analysis of mixed cultures, very important in industrial fermentations, became possible using metagenomic approaches. The novel molecular technologies such as metagenomic can be applied for the analysis of LAB metabolic potential. LAB isolates from African pearl millet slurries and the metagenomes of amylaceous fermented foods were screened for genes involved in probiotic functions and in starch metabolism (Turpin, Humblot & Guyot, 2011). Metagenomic approaches were also used to monitor changes in bacterial populations, metabolic potential, and overall

genetic features of the microbial community during the 29-day fermentation process of a traditional Korean food - kimchi (Jung *et al.*, 2011). Resulted metagenomic sequences were assigned to functional categories. Analysis of metabolic potential within the carbohydrate category indicated that the kimchi microbiome was enriched with the genes involved in mono-, di-, and oligosaccharide fermentation. The fermentation metabolism subcategory was associated mostly with various lactate fermentations and acetoin and butanediol metabolism. Moreover, metabolic genes involved in carbohydrate metabolism and fermentation generally increased as the kimchi fermentation progressed. Apart from the fermented food products, the current interest in the complex ecosystems is focused on the human gastro-intestinal tract for which more than three million bacterial genes have been characterised (Qin *et al.*, 2010; Methé *et al.*, 2012).

1.5. Novel Aspects of Metabolism Regulation in the Post-genomic Age

Regulation of transcription of DNA to mRNA in response to various signals is an essential mechanism for adaptation of microorganisms to changes of external or intracellular conditions. In Bacteria, transcription can be activated or repressed by various transcription factors (TFs) that recognize a specific *cis*-regulatory DNA elements, TF-binding sites (TFBSs) in the promoter regions of regulated genes. A set of genes or operons under direct control of the same TF is defined as a regulon. A set of all regulons in a certain organism forms a transcriptional regulatory network.

In recent years the number of genomic sequences of many dairy and non-dairy LAB have been dramatically increased. In each sequence, a part of the genes encode proteins dedicated to the regulation of transcription. Inspection of 30 sequenced LAB representatives indicates that the amount of such proteins encoded ranges from around 3.5% (*S. thermophillus*, *Lb. delbrueckii*, *Lb. helveticus*) to 7.5% (*Lb. plantarum*) of the entire proteome (Figure 4; (Ravcheev *et al.*, 2013a).

Also, the amount of total TFs varies significantly depending on the species within the LAB genomes, and ranges from 60-some (*S. thermophilus* and *Lb. helveticus*) to as many as 240 (*Lb. plantarum*). Within the 30 genomes of *Lactobacillales,* the putative TFs identified in the study of Ravcheev et al. (Ravcheev *et al.*, 2013a) are distributed between 49 protein families (on average 36 TFs per LAB genome) and approximately 90% of them belong to 24 major families with at least two members per genome. The greatest number of the TFs families representatives is recorded for the Xre family (298 TFs in total). Number of members of the Xre family reaches a dozen or even several dozen per genome (an average 19 per LAB genome). Other families are considerably less-represented, and those that have at least four representatives per genome include TetR, GntR, MarR, OmpR, LacI, LysR, MerR and AraC families (Figure 4; (Ravcheev *et al.*, 2013a). Some other systematic genome-wide analysis for transcriptional regulatory network reconstructions were performed for single LAB species including e.g. *Lb. plantarum* (Wels *et al.*, 2011) or *L. lactis* (de Jong *et al.*, 2012, 2013). In order to perform such sequence- and stored motifs-based reconstruction of gene regulatory networks several tools have been developed such as PEPPER, RegTransBase, PRODORIC, RegPrecise, FITBAR, RegAnalyst and MGcV (Overmars *et al.*, 2013) and references within. To discover new motifs toolboxes such as MEME (Bailey *et al.*, 2009), Tmod (Sun *et al.*, 2009), GIMSAN (Ng & Keich, 2008) and EXTREME (Quang & Xie, 2014) have been developed.

Despite an extensive research on analysis of available genomes, there is still a lack of comprehensive, experimental studies on regulatory networks operating in LAB. Most research on gene regulation in these bacteria focus on individual regulons mostly in model species such as either *L. lactis* subsp. *cremoris* MG1363 or in *L. lactis* subsp. *lactis* IL1403. In regard to carbohydrates metabolism, these studies have pointed out the role of the global (CcpA - catabolite control protein A) or specific regulators (ClaR, FruR, MalR, XylR from the RpiR,

DeoR, LacI, AraC family, respectively) in the regulation of different α- or β-glucosides, fructose, galactose, lactose, maltose, sorbose or xylose assimilation genes (reviewed by (Aleksandrzak-Piekarczyk, 2013; de Jong *et al.*, 2012). The CcpA protein is one of the components of a regulatory phenomenon defined as carbon catabolite repression (CCR), which also includes HPr, HPr kinase and the glycolytic enzymes fructose 1,6-bisphosphate and glucose-6-phosphate (Deutscher, 2008; Görke & Stülke, 2008). In large scale approaches such as transcriptomics and proteomics it has been shown that CcpA, besides the control of carbohydrate and energy metabolism, is involved in the regulation of tens of genes associated with membrane transport, nucleotide and nitrogen metabolism, protein biosynthesis and folding (Mazzeo *et al.*, 2012; Zomer *et al.*, 2006). In addition to abovementioned, CcpA-dependant CCR has been shown to impede expression of two operons involved in citrate metabolism in *E. faecalis* (Suárez *et al.*, 2011). Many genes undergo diverse regulation: via CcpA and via other TFs, as it has been shown by transcriptomic approach during the growth of *L. lactis* in the milk environment (de Jong *et al.*, 2013). The comparative systems biology approach revealed that phosphate plays a crucial role in the regulation of central metabolism and the uptake of glucose in *L. lactis* and *Streptococcus pyogenes* (Levering *et al.*, 2012). The comparison of both kinetic models revealed variations which can be explained by the differences in the phosphate levels of their natural environment. Recently, much effort has been put in research in the scope of proteolytic systems and amino acids biosynthesis regulation, especially in the *L. lactis* cells. In this bacterium, previous studies pointed out the superior role of the global transcriptional regulator CodY in the negative regulation of the proteolytic system components that is stimulated by the intracellular pool of branched chain amino acids (BCAAs) (den Hengst *et al.* 2005). Subsequent studies have been reported CodY-dependant regulation of several components of the proteolytic system in other LAB including *S. thermophilus* (Liu *et al.*, 2009), *Streptococcus pneumoniae* (Hendriksen *et al.*,

2008), *Streptococcus mutans* (Lemos *et al.*, 2008) and *O. oeni* (Ritt *et al.*, 2009) but not in lactobacilli. The latest research indicate that in lactobacillaceae and also enterococcaceae, leuconostocaceae, carnobacteriaceae, listeriaceae, exiguobacteria and bacillaceae, a separated from CodY, other proteolysis regulator may exist. This has been thoroughly tested in the *Lb. helveticus,* in which a novel BCARR protein, which functions to impede proteolysis gene expression in response to BCAAs has been found (Wakai & Yamamoto, 2013). No BCARR orthologs are present in the streptococcaceae, including lactococci, which have a CodY homolog. Amino acid assimilation is crucial for LAB that are generally auxotroph for amino acids. In addition to RNA structural switches, the control of sulfur amino acids metabolism have been shown also to depend on LysR-family transcriptional regulators such as: CmbR in *L. lactis* and CysR, HomR and MetR in *S. mutans* (Liu *et al.*, 2012 and references within). The global ArgR and AhrC regulons have been established by transcriptome analyses and show that both regulators are dedicated to the control of arginine metabolism in *L. lactis* (Larsen *et al.*, 2008). Interestingly, pneumococcal ArgR and AhrC, instead of being involved in the regulation of arginine biosynthesis and breakdown, as is the case in other bacteria, control the expression of genes dedicated to arginine and peptide uptake (Kloosterman & Kuipers, 2011). Moreover, AhrC was recently reported to have a role in virulence of pathogenic *S. pneumoniae* (Kloosterman & Kuipers, 2011) and *E.faecalis* (Frank *et al.*, 2013). Scarce global studies on modifications of LAB cell metabolism and involment of various regulatory mechanisms during amino acid shortage are available in the scientific literature. One of the few examples concerns the global transcriptom and proteom response of *L. lactis* during progressive isoleucine starvation. The global regulator CodY seemed specifically dedicated to the regulation of isoleucine supply whereas other regulations were related to growth rate and stringent response (Dressaire *et al.*, 2011).

1.6. Functional Genomics and Metabolism

1.6.1. Transcriptomics, Proteomics and Metabolomics

One of the great achievements of the recent molecular biology is the development of high throughput functional genomics approaches such as transcriptomics, proteomics or metabolomics. Transcriptomics have been used for the analysis of the LAB response to different environments concerning growth conditions, culturing regiments and various stresses (de Vos, 2011). A variety of studies, including functional genomics attempts, contributed to the discovery and further characteristics of respiratory metabolism in many species belonging to LAB (for recent reviews see Lechardeur *et al.*, 2011a; Pedersen *et al.*, 2012). Respiration is activated by the appropriate cofactors such as exogenous heme or heme and menaquinone. Moreover, some LAB species were found to use nitrate as terminal electron acceptor (Brooijmans, de Vos & Hugenholtz, 2009). Activation of electron transport chains lead to higher biomass production and increased robustness, which is beneficial for industrial applications (Pedersen *et al.*, 2012). Transcriptional analysis poited out the genes involved in oxygen respiration. Pedersen et al. have reported that the *ygfCBA* operon, encoding a putative transport system and regulator, is strongly induced by heme and is involved in heme tolerance and homeostasis (Pedersen *et al.*, 2008). Lechardeur et al. have reported that YgfC is a heme-responsive repressor that regulates the expression of the *hrtRBA* (formerly *ygfCBA*) operon in response to free heme molecules as physiological effectors and renamed YgfC as HrtR4 (heme-regulated transporter regulator) (Lechardeur *et al.*, 2011b). Recently, the cristal structure of the HrtR confirmed that it senses and binds a heme molecule to regulate the expression of the heme-efflux system responsible for heme homeostasis in *L. lactis* (Sawai *et al.*, 2012). A community transcriptomic approach revealed huge differences in expression profiles of genes between the human isolate *Lb. plantarum* grown

in the intestine and in laboratory media (Marco *et al.*, 2010). Moreover, similar expression profiles were observed in mice and human indicating common mechanisms of the intestinal adaptation. *Lb. plantarum* specifically adapted its metabolic capacity in the intestine for carbohydrate acquisition and expression of exopolysaccharide and proteinaceous cell surface compounds. The transcriptome response in the complex food ecosystems has been analysed for bacteria involved in yoghurt fermentation. *S. thermophilus* LMD-9 during growth in milk in the presence of *Lb. bulgaricus* overexpressed genes involved in aminoacid transport and metabolism as well as DNA replication (Goh *et al.*, 2011). The metatranscriptomic approach in respect to the yoghurt consortium showed that specific compounds and metabolic pathways are involved in the interactions between two strains (Sieuwerts *et al.*, 2010). Simillar approaches were also applied to complex communities developing in fermented foods such kimchi (Nam *et al.*, 2009) or sour dough (Weckx *et al.*, 2011).

The proteomic tools can also be used to better understand the metabolism of microorganisms in food. Recently, the proteomic approach were applied for quantitative analysis of bacterial enzymes released in Swiss-type cheese during ripening (Jardin *et al.*, 2012). This kind of approach was used to investigate the LAB response to different type of stress such as: acidic growth conditions (Nezhad, 2010) or low temperature (Garnier *et al.*, 2010). The recent modeling approaches of protein and mRNA stability based on transcriptome and proteome data of *L. lactis* increased the quantitative character of functional genomics tools (Dressaire *et al.*, 2009; Picard *et al.*, 2009).

The global metabolomics studies have not been reported frequently for LAB (de Vos, 2011), and might be illustrated by the analysis of folate-overproducing *Lb. plantarum* cells (Wegkamp *et al.*, 2010). Results from this analysis explained the growth rate reduction upon the overexpression of the folate gene cluster. In another study, the response of *L. lactis* to acid stress were analysed at

the metabolite level and complemented by performing a genome-wide transcriptome analysis (Carvalho *et al.*, 2013). These results allowed to determine the H+/lactate stoichiometry of lactic acid export and to propose a metabolic model accounting for the molecular mechanisms underlying the acid stress response in *L. lactis*.

1.6.2. Biolog Phenotype MicroArrays for Phenotypic Characterization of Microbial Cells

Phenotype MicroArray™ (PM) technology (Biolog Inc) is a system of cellular assays for the simultaneous, high-throughput screening of a large variety of phenotypes, which sufficiently complement the traditional genomic, transcriptomic and proteomic analysis. This technology allows to test nearly 2,000 microbial phenotypes (C, N, P, and S metabolism, pH growth range and regulation of pH control, sensitivity to NaCl and other ions, and sensitivity to chemical agents) and gain a comprehensive overview of pathway functions in a single experiment (Bochner, 2009). Despite the fact that LAB seem to be challenging to test using PM technique, in recent years a number of research work presenting the results of PM analysis on this group of microorganisms have been published. The most common use of the technology concerns the comparison of LAB cell lines that differ by a single gene mutation, thereby analyzing gene function. The example includes the assessing the role of the lactate dehydrogenase enzyme (LDH) on the general physiology of *L. lactis*, *E. faecalis*, and *S. pyogenes*. By screening a large variety of carbon sources available on PM arrays, it has been shown that the *ldh* deletion mutants are not able to utilize all carbon sources as efficiently as their cognate wild-types and that may be a reason of their growth rate-impairment observed in rich medium but not in chemically defined medium (Fiedler *et al.*, 2010). In another study, *S. mutants* and its mutant in the *liaS* gene encoding a cell-envelope stress-sensing histidine kinase (considered to be important for expression of virulence factors) were subjected to comprehensive PM analysis of about 2000

phenotypes (Zhang & Biswas, 2009). Compared to the parental strain, the *liaS* mutant was more tolerant to various inhibitors that target protein synthesis, DNA synthesis and cell-wall biosynthesis, whereas no significant difference in the carbon utilization panels was found between these two strains (Zhang & Biswas, 2009).

Other uses of PM technique include analyzing of naturally occurring microbial strains, analyzing of the biological properties of strains, analyzing of the effects of chemicals on cells, and employing it as a tool in bioprocess optimization. For instance, PM analysis was used to understand the bacterial phenotypic manifestations of environmental adaptation, which in turn may reflect on the technological processes (Di Cagno *et al.*, 2010). In this study, the comparison of a large variety of carbon sources metabolism was performed on 72 isolates of *Lb. plantarum* extracted from different raw vegetables and fruits. Based on various use of 27 carbon sources, eight clusters of *Lb. plantarum* isolates were formed, interestingly, not according to the original habitat (Di Cagno *et al.*, 2010). In another exemplary study, combined genomic, transcriptomic, and PM analysis were designed to understand the survival capacity in the complex sourdough ecosystem and role in the microbial community of the isolated from sourdough *L. lactis* strain (Passerini *et al.*, 2013a). Effective utilization of five carbon (C5) and six carbon (C6) sugars derived from lignocellulosic biomass would significantly improve the economic conversion of this material to biofuels and bioproducts. In the study of Lui et al. the PM analysis indicated that the *Lactobacillus buchneri* strain is able to metabolize a broad spectrum of carbon sources including various C5 and C6 monosaccharides, disaccharides and oligosaccharides, with better rates under anaerobic conditions, has a great tolerance to ethanol and other stresses, making it an attractive candidate for biomass conversion to various bioproducts (Liu, Skinner-Nemec & Leathers, 2007).

1.7. System Biology of LAB

Different types of modelling approaches have been successfully applied to monocultures of LAB species (Santos, de Vos & Teusink, 2013). Among them, kinetic models that are used to integrate experimental data of biochemical studies on transport process, kinetics of enzymes as well as flux and metabolites measurements such as NMR (Teusink, Bachmann & Molenaar, 2011). This strategy in *L. lactis* has been focused mostly on glycolysis starting from first trials (Hoefnagel *et al.*, 2002a, 2002b) to more recent studies (Voit, Neves & Santos, 2006; Andersen *et al.*, 2009; Oh, 2011; Levering *et al.*, 2012) Genome sequencing, genome analysis and their derived omic techniques (proteomics, transcriptomics, metabolomics, etc.) allow construction of genome-scale metabolic models (Teusink *et al.*, 2006; Pastink *et al.*, 2009; Santos *et al.*, 2009; Flahaut *et al.*, 2013), which will be useful in elucidating many cellular biochemical pathways. Coupling of genomic data with metabolic fingerprinting using high throughput techniques would further facilitate extraction of relevant biological information as in the case of citrate utilization and subsequent generation of flavour compounds (Bachmann *et al.*, 2009; de Bok *et al.*, 2011). The latest developments and future perspectives in modelling concern mixed cultures and complex ecosystems, for the recent review see, (Santos, de Vos & Teusink, 2013). This type of analyses has been applied for food consortia with the example of yoghurt consisting of *S. thermophilus* and *Lb. bulgaricus* (Sieuwerts, 2009) as well as for human gastro-intestinal tract (gut) microbiota (de Graaf *et al.*, 2010). Unravelling the physiology of multi-species microbial communities such as gut is a great challenge and will require combining of phylogenetic, metagenomic and meta-functional genomics data (Martins dos Santos, Müller & De Vos, 2010).

Furthermore, Systems Biology, which considers the study of microorganisms as integrated and interacting networks of genes, proteins, and biochemical reactions (Teusink, Bachmann &

Molenaar, 2011; de Vos, 2011), would be a powerful tool to decipher metabolism of LAB species.

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Figure captions

Figure 1. Pathways of glucose metabolism. A. Homofermentative pathway; B. Mixed-acid metabolism; C. Heterofermentative pathway; D. Leloir pathway. Reactions are catalyzed by the following enzymes: 1, glucokinase (GLK); 2, glucose-phosphate isomerase (GPI); 3,

phosphofructokinase (PFK); 4, fructose-bisphosphate aldolase (FBPA); 5, triose-phosphate isomerase (TPI); 6, glyceraldehyde-phosphate dehydrogenase (GAPDH); 7, phosphoglycerate kinase (PGK); 8, phosphoglycerate mutase (PMG); 9, enolase (ENO); 10, pyruvate kinase (PK); 11, lactate dehydrogenase (LDH); 12, pyruvate dehydrogenase (PDH); 13, pyruvate formate lyase (PFL); 14, acetaldehyde dehydrogenase (ACDH); 15, alcohol dehydrogenase (ADHE); 16, phosphotransacetylase (PTA); 17, acetate kinase (ACK); 18, α-acetolactate synthase (ALS); 19, α-acetolactate decarboxylase (ALD); 20, 2,3-butanediol dehydrogenase (BDH); 21, diacetyl reductase (DR); 22, glucose-6-P dehydrogenase (G6PDH); 23, 6-P-gluconate dehydrogenase (6PGDH); 24, ribulose-5-P-3-epimerase (RPPE); 25, D-xylulose-5P phosphoketolase (XPK); 26, galactokinase (GK); 27, galactose-1-P-uridylyltransferase (GPUDLT); 28, UDP-galactose-1-epimerase (UDPE); 29, phosphoglucomutase (PGM). (Please, reuse artwork from the previous edition, Chapter 1, page 5)

Figure 2. Citrate metabolism in *Lactococcus, Leuconostoc and Weissella species*. Key for the enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; ALS, α-acetolactate synthase; ADC, α-acetolactate decarboxylase; DAR, diacetyl acetoin reductase; BDH, 2,3-butanediol dehydrogenase; Tppi, thiamine pyrophosphate.

Figure 3. Diagram of the proteolytic systems of lactic acid bacteria. Location and targets of the different components are indicated.

Figure 4. Distribution of predicted transcription factors (TFs) in selected Lactobacillaceae (A) and Streptococcaceae (B) genomes. The least-represented TFs families (AbrB, AsnC, BirA, CcpN, CodY, ComK, CtsR, DtxR, Fis, Fur, GlnL, GutM, HrcA, HTH_11, IclR, LexA, NiaR,

NrdR, NrtR, PF04394, PurR, Rex, ROK, SdaR, SfsA, SorC, YobV) are not presented on the ideograms. Row data derive from (Ravcheev *et al.*, 2013b). Graphical presentation of these data has been developed with Circos (v0.64; Krzywinski *et al.*, 2009). Strains of presented *Lactobacillales* species: *L. lactis cremoris* (*Lactococcus lactis subsp. cremoris* SK11), *L. lactis lactis* (*Lactococcus lactis subsp. lactis* IL1403), *S. thermophilus* (*Streptococcus thermophilus* CNRZ1066), S. agalactiae (*Streptococcus agalactiae* 2603V/R) *S. uberis* (*Streptococcus uberis* 0140J), *S. equi* (*Streptococcus equi* MGCS10565), *S. dysgalactiae* (*Streptococcus dysgalactiae* GGS_124), *S. pyogenes* (*Streptococcus pyogenes* M1 GAS), *S. gallolyticus* (*Streptococcus gallolyticus* UCN34), *S. mutans* (*Streptococcus mutans* UA159), *S. suis* (*Streptococcus suis* 05ZYH33), *S. mitis* (*Streptococcus mitis* B6), *S. pneumoniae* (*Streptococcus pneumoniae* TIGR4), *S. gordonii* (*Streptococcus gordonii* CH1), *S. sanguinis* (*Streptococcus sanguinis* SK36), *L. sakei* (*Lactobacillus sakei* 23K), *L. casei* (*Lactobacillus casei* ATCC 334), *L. rhamnosus* (*Lactobacillus rhamnosus* GG), *L. delbrueckii* (*Lactobacillus delbrueckii* ATCC BAA-365), *L. acidophilus* (*Lactobacillus acidophilus* NCFM), *L. helveticus* (*Lactobacillus helveticus* DPC 4571), *L. johnsonii* (*Lactobacillus johnsonii* NCC 533), *P. pentosaceus* (*Pediococcus pentosaceus* ATCC 25745), *L. brevis* (*Lactobacillus brevis* ATCC 367), *L. plantarum* (*Lactobacillus plantarum* WCFS1), *L. fermentum* (*Lactobacillus fermentum* IFO 3956), *L. reuteri* (*Lactobacillus reuteri* JCM 1112), *O. oeni* (*Oenococcus oeni* PSU-1), *L. mesenteroides* (*Leuconostoc mesenteroides* ATCC 8293), *L. salivarius* (*Lactobacillus salivarius* UCC118).

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